#4

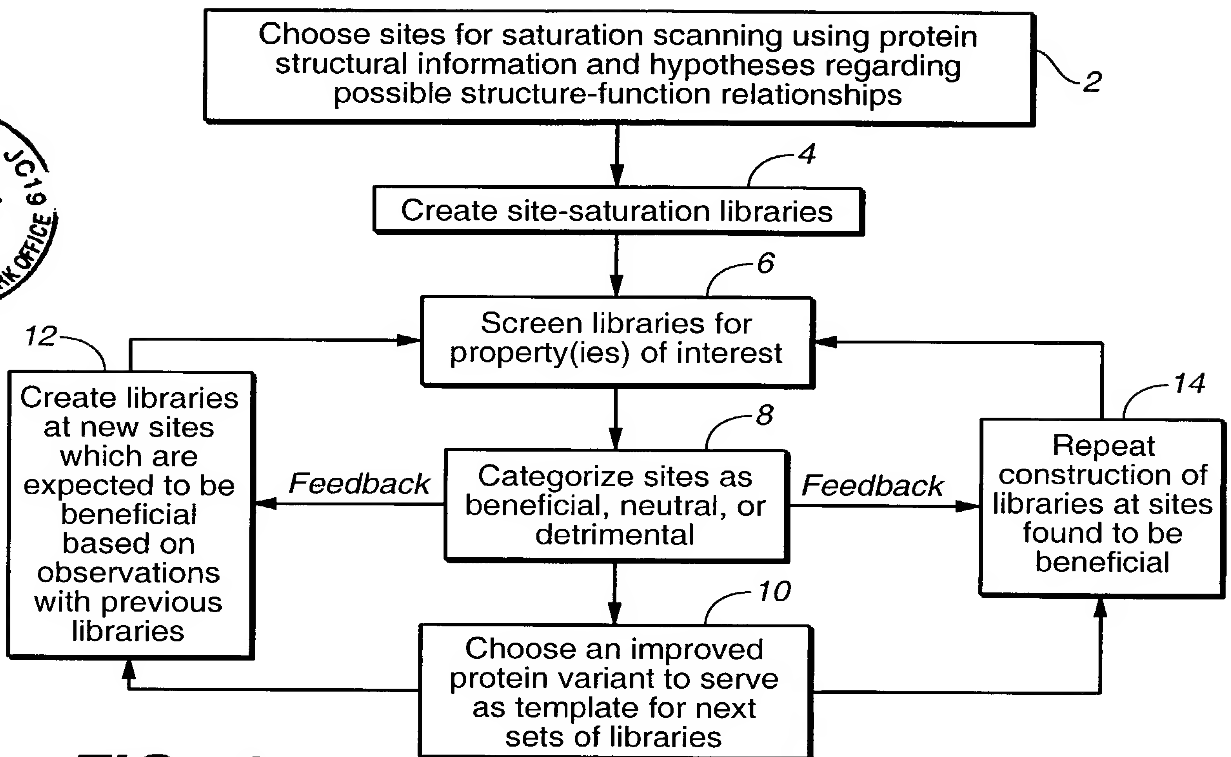
Choose sites for saturation scanning using protein structural information and hypotheses regarding possible structure-function relationships
2
4
Create site-saturation libraries
6
Screen libraries for property(ies) of interest
12
Create libraries at new sites which are expected to be beneficial based on observations with previous libraries
Feedback
8
Categorize sites as beneficial, neutral, or detrimental
Feedback
14
Repeat construction of libraries at sites found to be beneficial
10
Choose an improved protein variant to serve as template for next sets of libraries

FIG._1

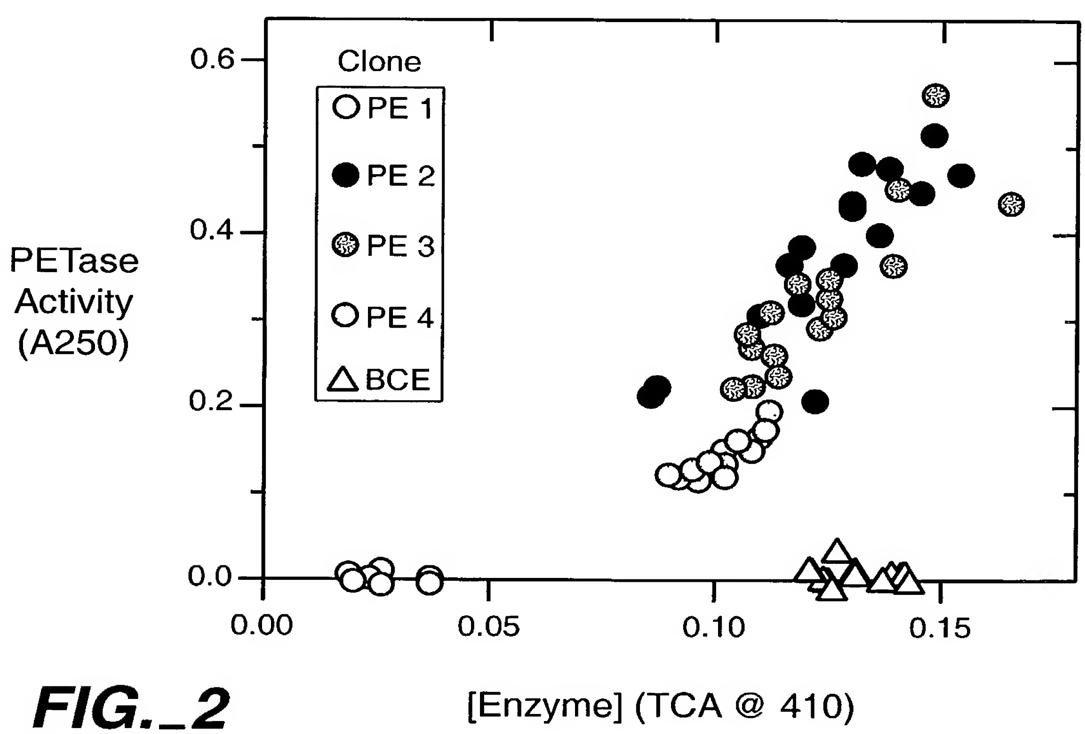
PETase Activity (A250)
0.6
0.4
0.2
0.0
Clone
PE 1
PE 2
PE 3
PE 4
BCE
0.00
0.05
0.10
0.15
[Enzyme] (TCA @ 410)

FIG._2

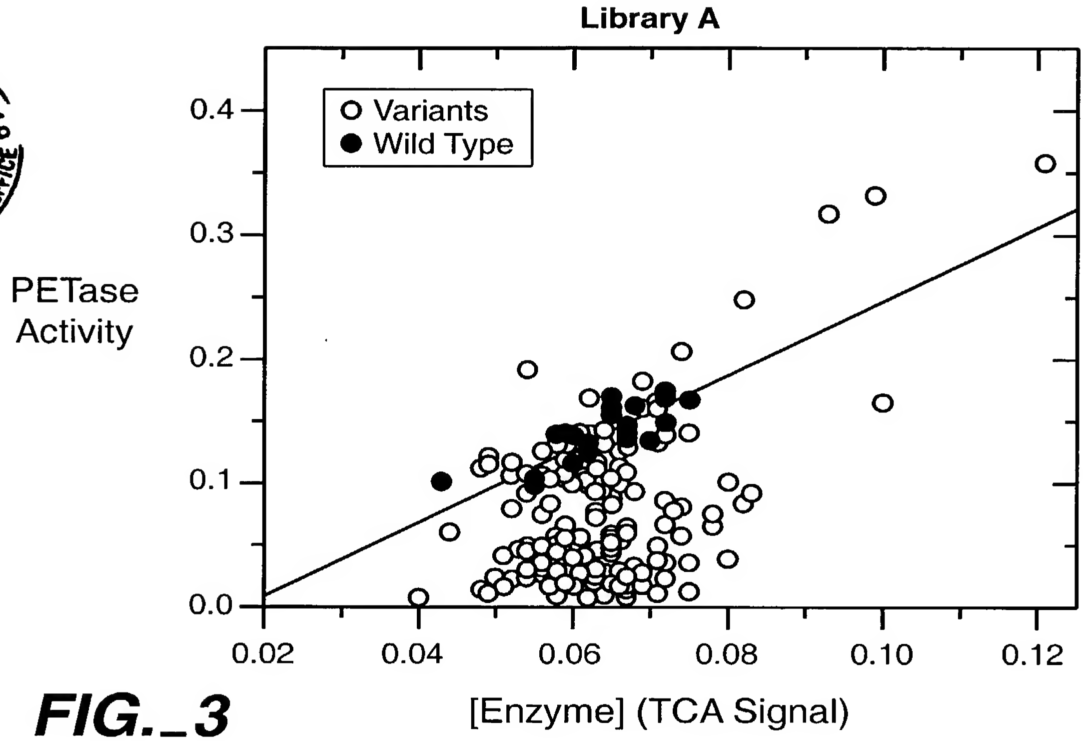
Library A
Variants
Wild Type
PETase Activity
0.4
0.3
0.2
0.1
0.0
0.02
0.04
0.06
0.08
0.10
0.12
[Enzyme] (TCA Signal)

FIG._3

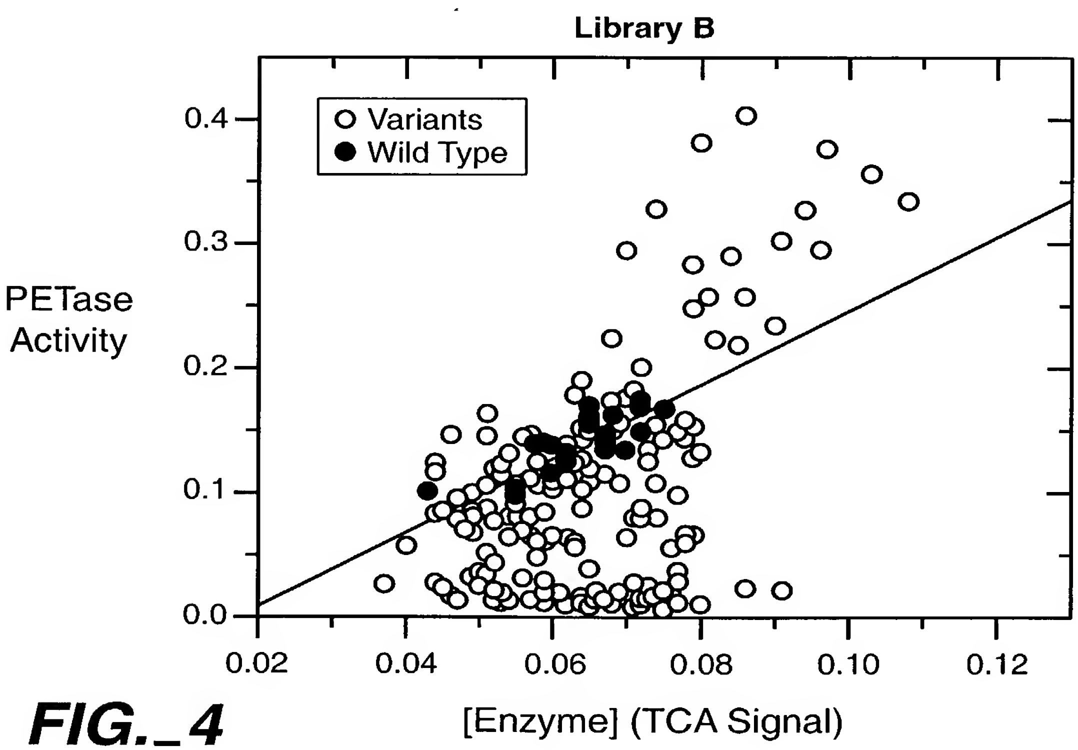
Library B
Variants
Wild Type
PETase Activity
0.4
0.3
0.2
0.1
0.0
0.02
0.04
0.06
0.08
0.10
0.12
[Enzyme] (TCA Signal)

FIG._4

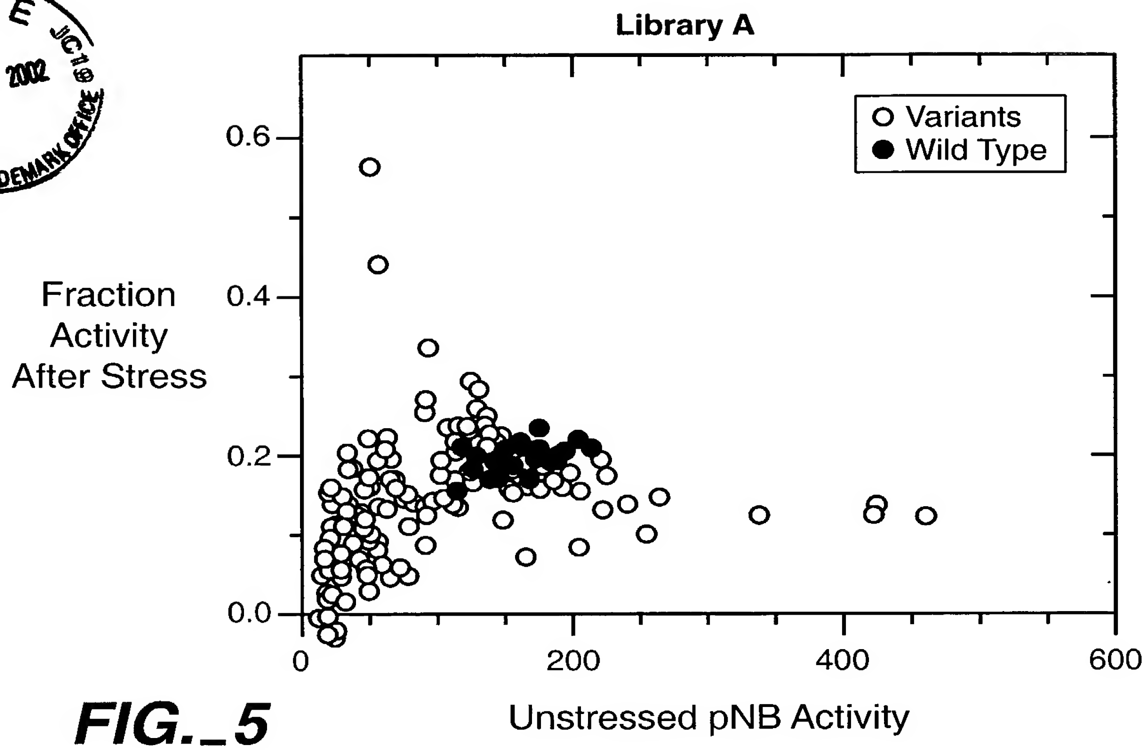
Library A
Variants
Wild Type
Fraction Activity After Stress
0.6
0.4
0.2
0.0
0
200
400
600
Unstressed pNB Activity

FIG._5

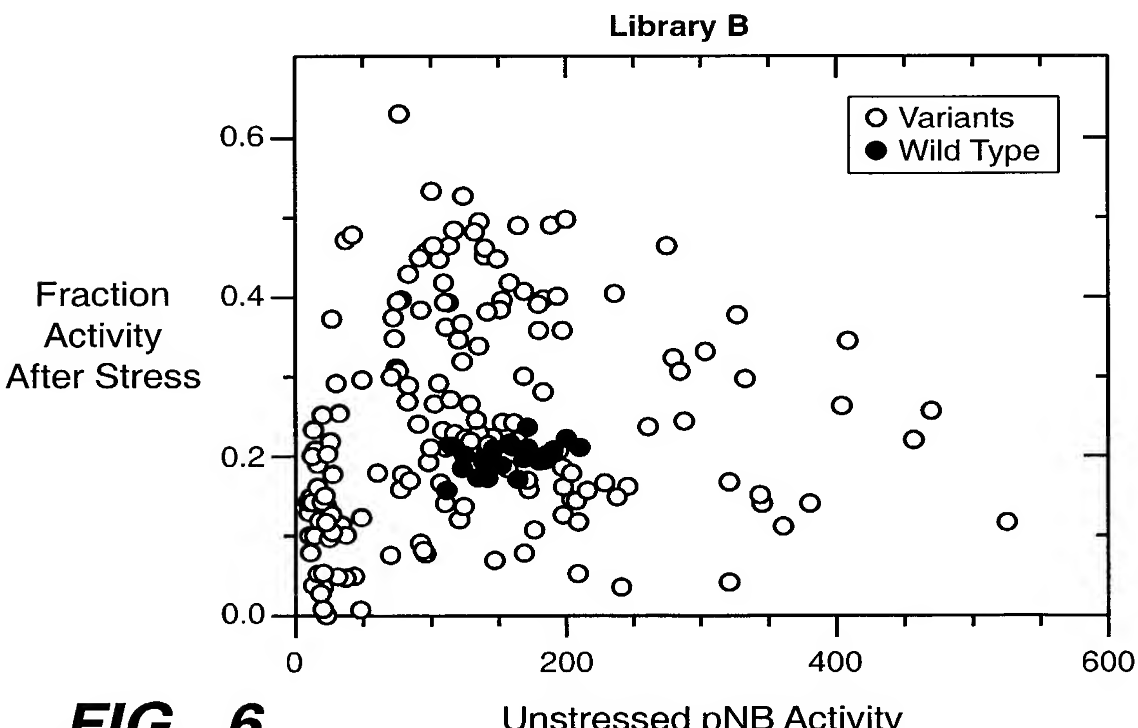
Library B
Variants
Wild Type
Fraction Activity After Stress
0.6
0.4
0.2
0.0
0
200
400
600
Unstressed pNB Activity

FIG._6

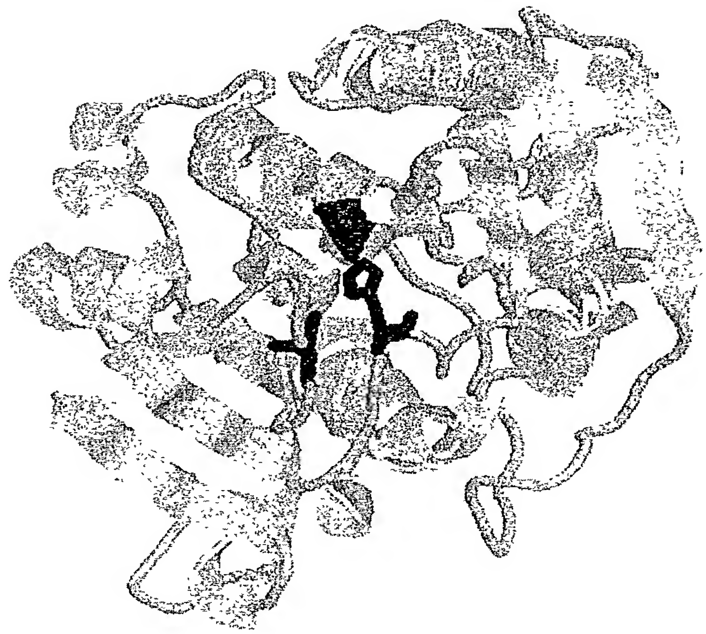

FIG._7

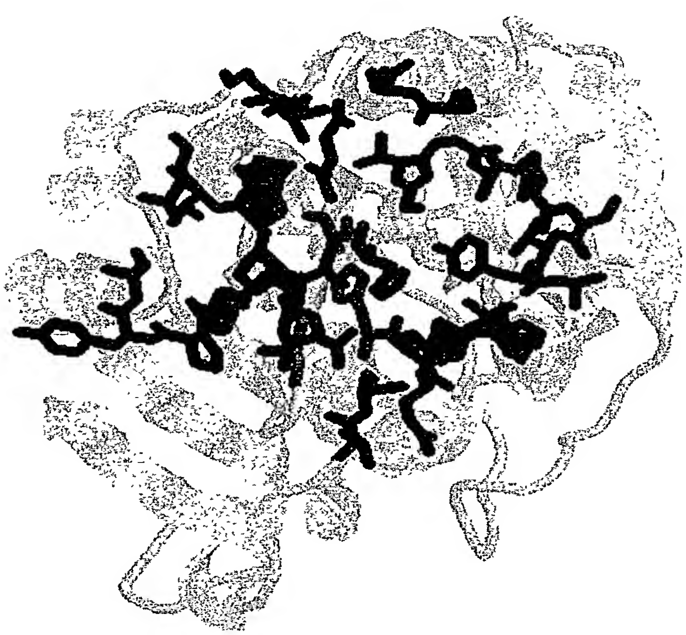

FIG._8

FIG._9

FIG._10

FIG._11

FIG._12

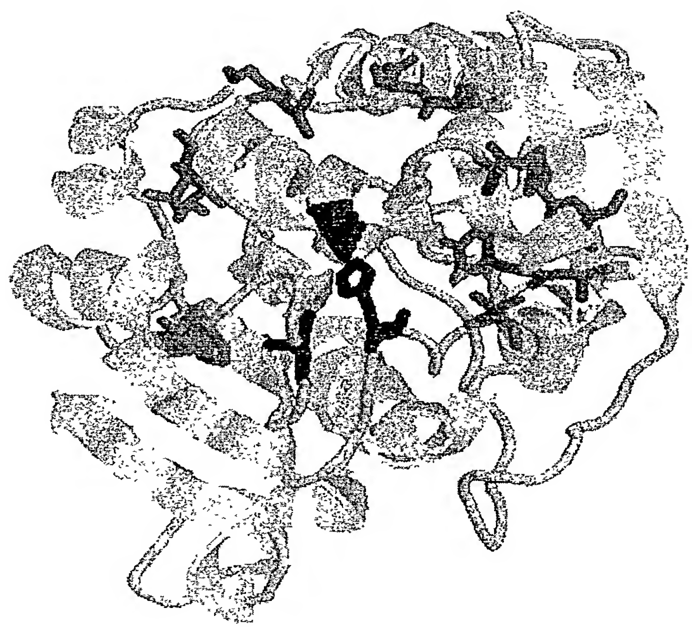

FIG._13

FIG._14

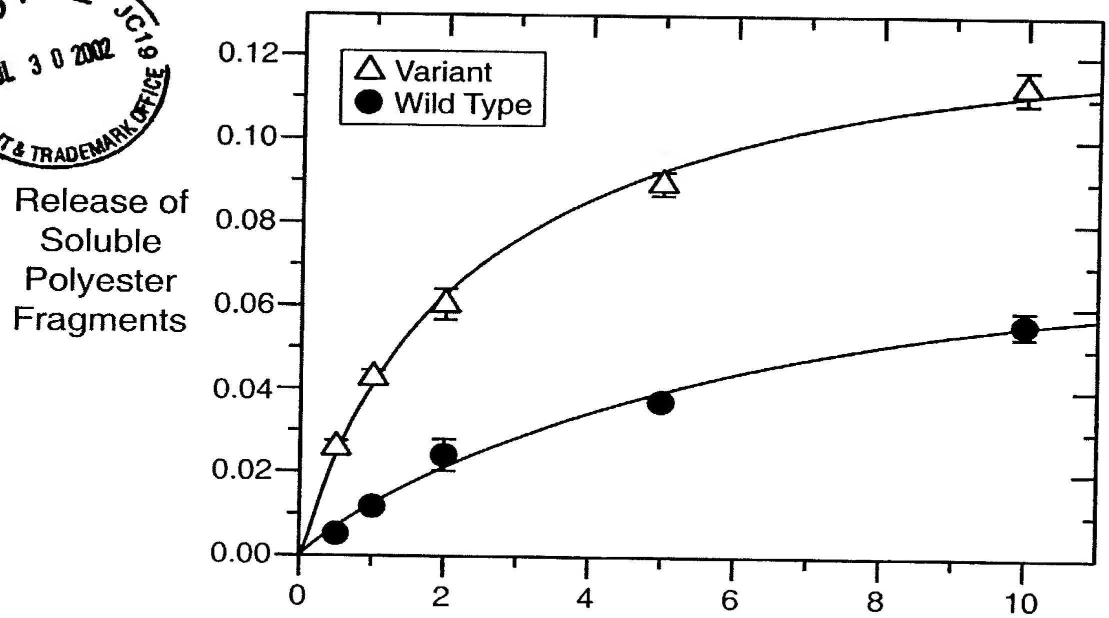
Variant
Wild Type
Release of Soluble Polyester Fragments
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
2
4
6
8
10
Enzyme Concentration

FIG._15

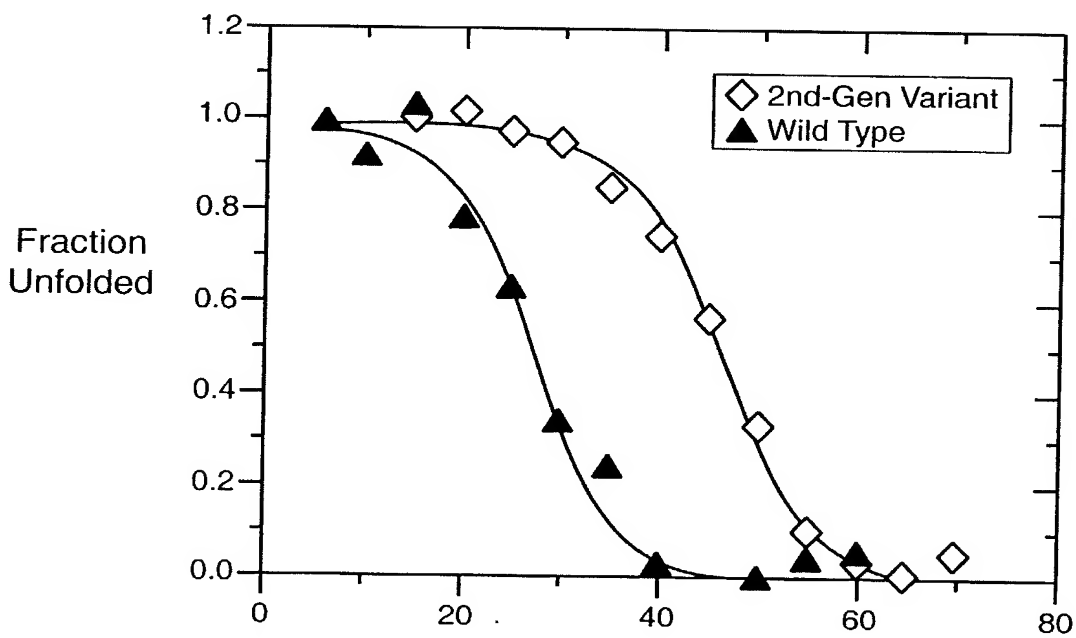
2nd-Gen Variant
Wild Type
Fraction Unfolded
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Temperature (°C)

FIG._16

```
TGGCGGCCTCTTGCCTGTCCGTCTGTGCCACTGTCGCGGC  40
GGCTCCCCTGCCGGATACACCGGGAGCGCCATTTCCGGCT  80
GTCGCCAATTTCGACCGCAGTGGCCCCTACACCACCAGCA 120
GCCAGAGCGAGGGGCCGAGCTGTCGCATCTATCGGCCCCG 160
CGACCTGGGTCAGGGGGGCGTGCGTCATCCGGTGATTCTC 200
TGGGGCAATGGCACCGGTGCCGGGCCGTCCACCTATGCCG 240
GCTTGCTATCGCACTGGGCAAGCCACGGTTTCGTGGTGGC 280
GGCGGCGGAAACCTCCAATGCCGGTACCGGGCGGGAAATG 320
CTCGCCTGCCTGGACTATCTGGTACGTGAGAACGACACCC 360
CCTACGGCACCTATTCCGGCAAGCTCAATACCGGGCGAGT 400
CGGCACTTCTGGGCATTCCCAGGGTGGTGGCGGCTCGATC 440
ATGGCCGGGCAGGATACGAGGGTGCGTACCACGGCGCCGA 480
TCCAGCCCTACACCCTCGGCCTGGGGCACGACAGCGCCTC 520
GCAGCGGCGGCAGCAGGGGCCGATGTTCCTGATGTCCGGT 560
GGCGGTGACACCATCGCCTTTCCCTACCTCAACGCTCAGC 600
CGGTCTACCGGCGTGCCAATGTGCCGGTGTTCTGGGGCGA 640
ACGGCGTTACGTCAGCCACTTCGAGCCGGTCGGTAGCGGT 680
GGGGCCTATCGCGGCCCGAGCACGGCATGGTTCCGCTTCC 720
AGCTGATGGATGACCAAGACGCCCGCGCTACCTTCTACGG 760
CGCGCAGTGCAGTCTGTGCACCAGCCTGCTGTGGTCGGTC 800
GAGCGCCGCGGGCTTTAA                       818
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FIG._17

```
TGGCGGCCTCTTGCCTGTCCGTCTGTGCCACTGTCGCGGCGGCTCCCCTGCCGGATACACCGG   63
MetAlaAlaSerCysLeuSerValCysAlaThrValAlaAlaAlaProLeuProAspThrPro

GAGCGCCATTTCCGGCTGTCGCCAATTTCGACCGCAGTGGCCCCTACACCACCAGCAGCCAGA  126
GlyAlaProPheProAlaValAlaAsnPheAspArgSerGlyProTyrThrThrSerSerGln

GCGAGGGGCCGAGCTGTCGCATCTATCGGCCCCGCGACCTGGGTCAGGGGGGCGTGCGTCATC  189
SerGluGlyProSerCysArgIleTyrArgProArgAspLeuGlyGlnGlyGlyValArgHis

CGGTGATTCTCTGGGGCAATGGCACCGGTGCCGGGCCGTCCACCTATGCCGGCTTGCTATCGC  252
ProValIleLeuTrpGlyAsnGlyThrGlyAlaGlyProSerThrTyrAlaGlyLeuLeuSer

ACTGGGCAAGCCACGGTTTCGTGGTGGCGGCGGCGGAAACCTCCAATGCCGGTACCGGGCGGG  315
HisTrpAlaSerHisGlyPheValValAlaAlaAlaGluThrSerAsnAlaGlyThrGlyArg

AAATGCTCGCCTGCCTGGACTATCTGGTACGTGAGAACGACACCCCCTACGGCACCTATTCCG  378
GluMetLeuAlaCysLeuAspTyrLeuValArgGluAsnAspThrProTyrGlyThrTyrSer

GCAAGCTCAATACCGGGCGAGTCGGCACTTCTGGGCATTCCCAGGGTGGTGGCGGCTCGATCA  441
GlyLysLeuAsnThrGlyArgValGlyThrSerGlyHisSerGlnGlyGlyGlyGlySerIle

TGGCCGGGCAGGATACGAGGGTGCGTACCACGGCGCCGATCCAGCCCTACACCCTCGGCCTGG  504
MetAlaGlyGlnAspThrArgValArgThrThrAlaProIleGlnProTyrThrLeuGlyLeu

GGCACGACAGCGCCTCGCAGCGGCGGCAGCAGGGGCCGATGTTCCTGATGTCCGGTGGCGGTG  567
GlyHisAspSerAlaSerGlnArgArgGlnGlnGlyProMetPheLeuMetSerGlyGlyGly

ACACCATCGCCTTTCCCTACCTCAACGCTCAGCCGGTCTACCGGCGTGCCAATGTGCCGGTGT  630
AspThrIleAlaPheProTyrLeuAsnAlaGlnProValTyrArgArgAlaAsnValProVal

TCTGGGGCGAACGGCGTTACGTCAGCCACTTCGAGCCGGTCGGTAGCGGTGGGGCCTATCGCG  693
PheTrpGlyGluArgArgTyrValSerHisPheGluProValGlySerGlyGlyAlaTyrArg

GCCCGAGCACGGCATGGTTCCGCTTCCAGCTGATGGATGACCAAGACGCCCGCGCTACCTTCT  756
GlyProSerThrAlaTrpPheArgPheGlnLeuMetAspAspGlnAspAlaArgAlaThrPhe

ACGGCGCGCAGTGCAGTCTGTGCACCAGCCTGCTGTGGTCGGTCGAGCGCCGCGGGCTTTAA    818
TyrGlyAlaGlnCysSerLeuCysThrSerLeuLeuTrpSerValGluArgArgGlyLeu*
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FIG._18